Opposing roles of *Nfkb2* gene products p100 and p52 in the regulation of breast cancer stem cells

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Abstract

Purpose: Nuclear factor-kappa B (NF-κB) signalling has been shown to regulate properties of breast cancer stem cells. However, the specific contribution of the non-canonical NF-κB pathway, components of which are elevated in aggressive breast cancer has not been addressed.

Methods: Through shRNA silencing of the Nfkb2 gene, the role of p100/p52 in 4T1 and N202.1A cell lines were assessed by NF-κB reporter, invasion, tumoursphere and orthotopic transplantation assays. The processing of p100 into p52 was also inhibited with a p97 ATPase inhibitor, NMS-873, and its effects on tumoursphere formation was assessed.

Results: Knockdown of Nfkb2 led to opposing changes in NF-κB dependent transcription. NF-κB activity was elevated in 4T1 cells and this resulted in increased motility, cancer stem cell (CSC) activity and tumourigenicity in vivo. Conversely, depletion of Nfkb2 in N202.1a cells decreased NF-κB activity, CSC properties and tumourigenicity in vivo. By selectively over-expressing the p52 subunit in Nfkb2 depleted cells, we found that the increased malignancy in 4T1 cells could not be reverted in the presence of p52 whereas the decreased tumourigenicity of N202.1a cells could be rescued by p52. These results indicate that p100 and its subunit p52 have opposing effects on breast CSC activity. Accordingly, inhibition of an upstream regulator of p100 processing was effective in reducing tumoursphere formation of N202.1A and SKBR3 (ErbB2 ^{HIGH}) cells without aggravating that of 4T1 and MDA-MB-231 (ErbB2^{LOW}) cells.

Conclusion: These findings indicate that inhibiting the processing of p100 may be a potential therapeutic strategy to suppress CSC activity in a subset of breast tumours.

Keywords: breast cancer, Nfkb2, NF-KappaB, cancer stem cell, ErbB2, p97 ATPase

Introduction

Even though breast cancer predominantly affects women, it is the second most common form of cancer globally [1-4]. Significant advances have led to improved survival rates but the reality is that breast cancer is a heterogeneous disease which comprises of varying subtypes [5, 6]. While prognoses have improved for certain subtypes, some remain less adequate. This is mainly due to therapeutic resistance, metastasis and tumour relapse, which can be attributed at least in part to a subpopulation of cells with increased tumor initiating potential, termed cancer stem cells.

The existence of cancer stem cells (CSCs) were first shown in hematopoietic malignancies [7, 8] and more recently, the presence of CSCs in breast tumours represented the first indication of CSCs in solid tumours [9]. The CSC hypothesis states that cells within a tumour are not equal in their tumorigenic potential. Evidence suggests that within this hierarchy, it is the CSCs that fuel the growth of a tumour resulting in therapeutic resistance, disease relapse and the seeding of metastases in a range of cancer subtypes [10, 11]. CSCs have been shown to utilize and depend on distinct signalling pathways from non-CSCs and this suggests a possibility for specific therapeutic targeting to eradicate CSCs [12-14].

One such candidate pathway involves NF-κB dependent transcription. NF-κB signalling has been shown to promote breast CSC properties [35] and to result in EMT and metastasis in animal models of breast cancer [28-30]. The NF-κB proteins are transcription factors which were initially identified as regulators of the immune system. There are five different subunits which can form homo- or heterodimers to regulate the expression of genes. Namely, these are RelA (p65), RelB, c-rel, p50 and p52. All of these subunits have a conserved Rel homology domain

which facilitates DNA binding and dimerization. However, p50 and p52 differ from the Rel proteins in two ways. Firstly, they lack trans-activating domains which are important for initiating transcription. Secondly, p50 and p52 are translated as precursor proteins p105 and p100 respectively. These precursor proteins are inhibitor of NF-κB (IκB) proteins characterized by the presence of ankyrin repeats at the C-terminus. These motifs are removed upon partial proteasomal processing into p50 or p52 respectively. Apart from p105 and p100, other IκB proteins include IκBα, IκBβ, IκBε and Bcl-3. The IκB proteins function by masking the nuclear localization signal of NF-κB subunits and thus retain them in the cytoplasm. There are two main NF-κB pathways, namely, the canonical and alternative pathways. Transcriptional activity induced by canonical NF-κB signalling involves RelA-p50 dimers whereas the alternative pathway is dependent on RelB-p52 dimers. The alternative pathway, which is associated with constitutive NF-κB activity in normal and tumour cells is maintained through a signalling cascade involving NF-κB inducing kinase (NIK) and IκKα dimers, resulting in p100 phosphorylation and processing into p52. The loss of ankyrin domains from the C-terminus of p100 then allows translocation of RelB-p52 heterodimers into the nucleus [15, 16]. P100 also actively shuttles between the nuclear and cytoplasm and plays an inhibitory role by sequestering other NF-κB subunits in the nucleus [17, 18]. The processing of p100 into p52 has also been recently shown to depend on the ATPase activity of p97 and this presents a critical regulation point where the alternative pathway can be inhibited [19]. Taken together, the roles of the Nfkb2 gene which encodes both the p100 inhibitory subunit and p52 transcription factor is likely to be complex and context dependent.

As breast cancers are scrutinized further into more distinct subtypes [20], it is likely that more specific therapies will lead to more effective targeting of each particular subtype with less

side effects. The canonical NF-κB pathway has been shown to be important for tumorigenesis and CSC properties especially in estrogen receptor (ER) negative and ErbB2 driven tumours [21-24], yet the alternative pathway is also known to play a key role in driving oncogenic pathways such as inducing cyclin D1 expression [25, 26]. Aberrant levels of alternative NF-κB pathway components in human breast cancer tissue have been described [27, 28]. However, since p100 and p52 are known to play opposing roles in the regulation of the alternative NF-κB pathway, we set out to determine whether certain breast cancers may respond differentially to silencing of the Nfkb2 gene, and whether this acts on breast CSC viability. Interestingly, we found that depletion of *Nfkb2* has context dependent effects on 4T1 ($ErbB2^{low}$) and N202.1A ($ErbB2^{high}$) mammary cancer cell lines respectively. NF-κB activity was found to be differentially affected in these cell lines upon *Nfkb2* silencing and this led to corresponding phenotypes associated with cancer stem cell properties and activity. While our results corroborate previous findings where Nfkb2-mediated transcription was shown to be important for breast cancer stem cell activity [29], we provide evidence that selective targeting of p52 rather than upstream Nfkb2/p100 expression may have a more beneficial therapeutic potential in the in vivo setting.

Materials and Methods

Reagents and antibodies

Plasmid containing human p52 sequence was a kind gift from Professor Neil Perkins (Newcastle University). This HA-tagged p52 sequence was excised by XbaI-PstI digestion and ligated into the cloning site of P305 lentiviral plasmid. (Dr. Riccardo Brambilla, Cardiff University). NF-κB luciferase assays were carried out using the 3x κB luciferase reporter plasmid (Professor Ron Hay, University of St. Andrews) and pcDNA3.1-LacZ plasmid (Invitrogen) was used as control for transduction efficiency. Packaged lentiviral vectors encoding shRNA against *Nfkb2* (NM_019408) or non-target (NT) (SHC002V) control sequences were obtained from Sigma. Antibodies for Western blotting against β-actin (Abcam, Ab8227), Lamin A/C (CST 4777), p65 (CST 8243), p105/p50 (eBioscience 14-6732-81) and p100/p52 (SantaCruz, SC-7386) were used according to manufacturer's instructions. For flow cytometry experiments, CD29 (BD Biosciences, 562155), CD61 (eBioscience, 13-0611-81), CD44 (Biolegend, 103007) and CD24 (Biolegend, 311106) antibodies were used.

Cell culture

4T1 cells [30] were obtained from Dr. Robin Anderson (University of Melbourne) and N202.1A cells [31] were a kind gift from Dr. Pier-Luigi Lollini (Sezione di Cancerologia, Italy). 4T1 and SKBR3 cells were cultured in RPMI medium with 10% fetal bovine serum (FBS). N202.1A and MDA-MB-231 cells were cultured in DMEM medium that is supplemented with FBS at 10%. All media were also supplemented with 2mM L-glutamine and 50units/ml penicillin-streptomycin. For generation of stable cell lines, cells transduced with respective lentivirus were then cultured in 4ug/ml puromycin for 4T1 cells and 1ug/ml puromycin for

N202.1a. For BAY 11-7082 (Cayman Chemical) and NMS-873 (Apexbio) experiments, mammary cancer cells were maintained with 1μM BAY 11-7082 or NMS-873 throughout the time period of the assay.

NF-κB luciferase reporter assays

Cells were seeded in clear bottom black well plates and transfected 24 hours later with NF-κB reporter plasmid along with pcDNA3.1-LacZ as control for transfection efficiency using Lipofectamine 2000 (Invitrogen). After 48 hours, cells were lysed with Glo-lysis buffer (Promega). Luciferase and LacZ activity was measured after adding Bright-Glo (Promega) and Beta-Glo (Promega) substrates according to the manufacturer's instructions. Resulting luminescence were measured using a Flurostar Optima plate reader (BMG Labtech).

Transwell migration assay

Cells were dissociated into single cell suspension and seeded into each 24-well cell culture insert with 8um pores (BD Biosciences). Cells were seeded with respective medium containing 0.1% FBS at a density of 25,000 cells/chamber. Migration was stimulated by a serum gradient. After 24 hours, cells were fixed with ice cold 70% ethanol. Cells on the upper surface were then removed mechanically and the membranes were stained with haematoxylin and eosin for visualization.

Mammosphere forming assay

Cells were dissociated into single cell suspensions and re-suspended in mammosphere culture medium (MEBM, Lonza) supplemented with B27, 5mg/ml insulin, 20ng/ml Epidermal growth factor, 1mg/ml hydrocortisone and 0.0008% v/v β-mercapthoethanol. Cells were seeded

at a density of 500cells/well in ultra-low attachment 96-well plates (Corning) and incubated for 7 days. Passaging was achieved by dissociating mammospheres into single cell suspensions and re-plating under similar conditions.

qRT-PCR analysis

Isolation of RNA from cells was carried out using the RNeasy Mini Kit (Qiagen, Sussex, UK) according to manufacturer's instructions. Then, cDNA was synthesized from RNA samples using RevertAID Premium Reverse Transcriptase (Fermentas). SYBR Green reagent (Invitrogen) was used in the reaction mastermix and thermal cycling was carried out with a Step One Plus Realtime PCR System (Applied Biosystems). Cycling conditions used for all reactions consisted of; initial denaturation for 10 minutes at 95ºC, followed by 45 cycles of 95ºC for 15 seconds and 60ºC for 1 minute. List of primers used are: GAPDH F-CACAGTCAAGGCCGAGAATG, GAPDH R-TCTCGTGTTCACACCCATC, SNAIL F-TGAGAAGCCATTCTCCTGCT, SNAIL R-CTTCACATCCGAGTGGGTTT, SLUG F-TCTGCAGACCCACTCTGATG, SLUG R-AGCAGCCAGACTCCTCATGT, TWIST1 F-CAGGCCGGAGACCTAGATGTCAT, TWIST1 R-TGCCCCACGCCCTGATTCTTG, TWIST2 F-CTTCTCCGTGTGGCGCAT, TWIST2 R-CCGCATCCAGTTCCGCAT, ZEB2 F-CCGGGAGCTGTTTCTTCG, ZEB2 R-CGCAGGCTCGATCTGTGA, ECADHERIN F-CAGATGATGATACCCGGGACAA, ECADHERIN R-GGAGCCACATCATTTCGAGTCA, NCADHERIN F-AGGACCCTTTCCTCAAGAGC, NCADHERIN R-ATAATGAAGATGCCCGTTGG. C_T values obtained were processed using the $2^{-\Delta\Delta Ct}$ relative quantification method [32].

Western Blotting

Protein lysates were collected from cultured cells by lysing with RIPA buffer (150mM sodium chloride, 1% v/v Nonidet-P40, 0.1% w/v sodium dodecyl sulphate, 0.5% w/v sodium deoxycholate, 50mM Tris pH8) with the addition of complete mini protease inhibitor tablets (Roche) and phosphatase inhibitors (10mM sodium fluoride, 1mM sodium orthovanadate, 10mM sodium pyrophosphate). Proteins were then separated by SDS-PAGE and immune-blotted for detection of proteins.

Flow cytometry analysis

Cells were reconstituted in single cell suspensions and incubated with antibodies for 20 minutes at 4^0C. Flow analysis was carried out on a BD FACSCanto cell analyzer.

Orthotopic transplantation of mammary cancer cells and tumour measurements

For transplantation experiments, BalbC and NOD/SCID-BalbC mice of six to eight weeks of age were obtained from Charles River Laboratories. Maintenance, breeding and scientific procedures involving animals were carried out according to the guidelines set by the U.K. Home Office Regulations Animals (Scientific Procedures) Act 1986. Orthotopic cell transplants were carried out on mice between 8-12 weeks of age. For 4T1 transplants, cells were dissociated into single cell suspension and 10,000 cells were injected into the abdominal mammary glands. For N202.1a transplants, 50,000 cells were injected into NOD/SCID-BalbC mice.

Statistical analyses

Data are presented as means ±SEM. For statistical analyses, two-tailed unpaired student's t-test was used and the threshold for significance was $p<0.05$. For tumor growth curves, ANCOVA test was used to determine significance.

Results

Knockdown of Nfkb2 led to elevated basal NF-κB activity in 4T1 cells but decreased basal NF- κB activity in N202.1a cells

Through the analysis of The Cancer Genome Atlas (TCGA) breast datasets, we found elevated expression of NFKB2 transcripts in invasive breast carcinomas relative to normal breast tissue (Fig. 1a). This suggests a role for NFKB2 dependent signalling in advanced breast cancer. Consequently, we went on to address the direct contribution of *Nfkb2* to the tumourigenic properties of two invasive mammary cancer cell lines, N202.1A and 4T1, by shRNA knockdown. NF-κB activity has previously been reported to be associated with ErbB2 levels in breast cancer [26,27], and so as expected, the N202.1A cell line which was derived from an ErbB2 (Neu) over-expressing mammary tumour had concomitantly elevated nuclear p65 levels determined by western blot analysis compared to the ErbB2^{LOW} mammary cell line 4T1 (Figure 1b). In contrast however, the relative levels of nuclear p100/p52 and the ratio of p52 to p100 was higher in 4T1 cells relative to N202.1A cells. After silencing *Nfkb2,* the nuclear p52 to p100 ratio, as well as the nuclear to cytoplasmic p52 ratio, were further increased in 4T1 cells but decreased in N202.1A cells (Fig. 1b-1d) while no significant changes in the levels of p105, p50 or p65 were observed in both nuclear and cytoplasmic fractions upon *Nfkb2* knockdown. These relative changes in p52/p100 correlated with basal NF-κB activity as indicated by NF-κB reporter assay (Fig 1e). The different outcomes in activation of NF-κB in the cell lines examined could be due to varying levels of upstream signals which affect basal processing of p100 into p52. Irrespective of the underlying mechanism for this difference however, it is clear that perturbation of p100/p52 levels affect basal NF-κB activity and this demonstrates the contribution of non-canonical NF-κB pathway proteins towards overall basal NF-κB activity in

these mammary tumour cell lines. Given the presumptive role for NF-κB signalling in tumour growth and malignancy, we set out to evaluate whether the differential effects on NF-κB activity mediated by *Nfkb2* suppression affected cancer associated phenotypes in these cell lines.

Knockdown of Nfkb2 exclusively increased the motility of 4T1 cells and this increase correlated with induction of EMT

We therefore examined the effects of *Nfkb2* knockdown on the proliferative potential of mammary cell lines. Despite the previously described association between p52, cyclin D1 and proliferation in transformed cells [25], we did not observe any effects on the growth rate of cells (Online Resources 1a-b). We also assessed the consequences of *Nfkb2* knockdown on the colony forming potential of these cell lines but no significant differences were observed in the number of colony forming units (Online Resources 1a-c). A characteristic of tumour progression to malignancy is the phenotypic transition of a sub-population of tumour cells to become more motile and invade surrounding tissues. We addressed the role of p100/p52 in the regulation of these phenotypes *in vitro* through transwell migration assays. The loss of p100/p52 increased the motility of 4T1 cells but had no effect on N202.1A cells (Fig. 2a, Online Resource 2). Motility was unaffected in N202.1A cells despite decreases in NF-κB activity (Fig. 1e), suggesting that the alternative NF-κB pathway is not essential for motility in these cells. As the increase in motility of 4T1 cells correlated with an increase in basal NF-κB activity when *Nfkb2* was silenced (Fig. 1e), we wanted to address whether the increase in motility was dependent on NF-κB activity. Treatment of 4T1 Nfkb2 kd cells with the NF-κB inhibitor BAY 11-7082 substantially decreased the motility of these cells to a level that was lower than untreated non-

target (NT) control (Fig. 2b). Correspondingly, treatment of 4T1 NT cells with BAY 11-7082 also decreased motility to a level that was comparable to BAY 11-7082 treated 4T1 Nfkb2 kd cells (Fig. 2b). This implied that NF-κB activation was required not only for the enhanced motility induced by silencing *Nfkb2* in 4T1 cells, but also the inherent motility of these cells. In order to further dissect whether the loss of either p100 or p52 was responsible for the increase in motility of 4T1 Nfkb2kd cells, we re-expressed p52 in these cells but this failed to rescue the increase in motility due to silencing of *Nfkb2* (Fig. 2c). This suggested that it was the loss of p100 that was most likely responsible for the increase in motility of 4T1 Nfkb2kd cells.

Since NF-κB activation is capable of inducing epithelial to mesenchymal transition (EMT) and EMT can confer cancer cells with increased migratory properties [33], we were interested in determining whether 4T1 Nfkb2kd cells acquired an EMT phenotype. The transcript levels of the EMT-inducing transcription factor Zeb2 were increased 1.6 fold in 4T1 Nfkb2kd cells compared to controls (Fig. 2d). Accordingly, we observed a decrease in levels of E-cadherin and an increase in levels of N-cadherin relative to NT controls, which is characteristic of the EMT process.

The changes in basal NF-κB activity as a result of *Nfkb2* knockdown correlated with the changes in CSC properties of 4T1 and N202.1a cells respectively

The NF-κB pathway has been implicated in the regulation of breast CSC maintenance and proliferation [21, 22, 29]. In view of that, we wanted to determine whether p100/p52 had a role in governing such properties. When we analyzed the levels of putative breast CSC (i.e. $CD29^{hi}CD61^{+}$ cells) [34], a significant increase in the proportion of CSCs was observed for 4T1

cells (Fig. 3a and 3b) and on the other hand, CSC numbers were decreased by more than two fold for N202.1A cells (Fig. 3a and 3c). These changes led us to address CSC phenotypes by employing tumoursphere formation assays in serial passage. In serial passage there was a two fold increase in the proportion of tumourspheres when *Nfkb2* was silenced in 4T1 cells (Fig. 4a) while in N202.1A cells, a significant decrease in tumourspheres was observed following downregulation of Nfkb2 (Fig. 4a). Consequently, we tested the dependence on NF-κB activity of respective tumoursphere forming potentials for 4T1 and N202.1A cell lines through inhibition with BAY 11-7082. Notably, BAY 11-7082 treatment abolished the tumoursphere forming potentials of both cell lines to almost undetectable levels (Fig. 4b and 4c). This shows that the inherent tumoursphere forming potential of 4T1 and N202.1A cells upon *Nfkb2* silencing are dependent on NF-κB signalling. Over-expression of p52 in 4T1 Nfkb2 kd did not rescue the increased tumoursphere forming potential relative to 4T1 NT cells (Fig. 4d) further suggesting a role for un-processed p100 in 4T1 cell tumourigenesis. However, overexpression of p52 could rescue the decrease in sphere forming efficiency of N202.1A Nfkb2 kd cells (Fig. 4e), demonstrating a role for p52 in positively regulating tumoursphere forming potential.

Silencing of Nfkb2 had opposing effects on the tumorigenic potentials of 4T1 and N202.1a cells respectively in vivo

These changes in CSC marker expression and mammosphere forming potential implicate the involvement of p100/p52 in the regulation of tumour initiating properties. Furthermore *in vitro* experiments indicated that 4T1 cells exhibited more aggressive phenotypes when p100/p52 was depleted. To confirm these findings *in vivo* 4T1 NT and Nfkb2 kd cells were transplanted

into the mammary fat pads of recipient mice. 4T1 Nfkb2 kd cells exhibited enhanced growth kinetics (Fig. 5a). Although 4T1 Nfkb2 kd tumours were growing relatively faster than 4T1 NT tumours, we did not observe any differences in the proliferation or the number of apoptotic cells between the two cohorts, as determined by Ki67 and cleaved caspase-3 immuno-staining respectively (Fig. 5b-d). This correlated with our findings in vitro (Online Resource 1), suggesting that the difference in tumour growth may be due to increased numbers of tumour stem/progenitor cells within the 4T1 Nfkb2 kd population.

Concordantly, the decrease in N202.1A CSC activity observed in vitro correlated with a decrease in rate of tumour growth of N202.1A xenografts lacking *Nfkb2* (Fig. 5e). Similarly, we did not observe any significant difference in the rate of proliferation or apoptosis between the N202.1a tumor cohorts as indicated by Ki-67 and cleaved caspase-3 staining (Fig. 5f-h). It is possible therefore that the retardation in N202.1a Nfkb2 kd tumor growth was due to decreased stem/progenitor cells at the onset of transplant rather than a more general effect on proliferation or cell death.

NMS-873 treatment reduced the sphere forming potential of N202.1A and SKBR3 cells

Based on the contrasting effects observed in 4T1 and N202.1A cells and the potential role for un-processed p100 in these responses, we speculated that inhibition of p100 processing might be a more viable therapeutic strategy to avoid the loss of p100 and reduce p52 levels concurrently. For that reason, we inhibited an upstream regulator of p100 processing, the p97/VCP ATPase [19], using NMS-873, a potent and selective inhibitor of this ATPase. In serial passage tumoursphere cultures, we observed a decrease in the sphere forming potential of

N202.1A cells after NMS-873 treatment (Fig. 6A), which is consistent with our observations of *Nfkb2* silencing in this cell line (Fig. 4A). Importantly, although there is no decrease in the sphere forming potential of 4T1 cells in the presence of NMS-873 (Fig. 6A), these cells did not exhibit increased sphere forming potential, as observed in 4T1 Nfkb2 kd cells (Fig. 4A). These results also suggest that ErbB2^{HIGH} cells may be more sensitive to p97 inhibition. We expanded these observations into two human breast cancer cell lines, MDA-MB-231 (ErbB2^{LOW}) and SKBR3 (ErbB2^{HIGH}), and similarly, NMS-873 was only effective in reducing sphere formation in SKBR3 cells (Fig. 6B). We also examined the expression of CSC markers, CD24 and CD44 upon NMS-873 treatment in these cell lines. In MDA-MB-231 cells, no significant changes in the $CD44^{+}CD24^{-}$ or $CD44^{+}CD24^{+}$ populations were observed (Online resource 3). As for SKBR3 cells, a $CD44^{+}CD24^{-}$ CSC population is not observed in this cell line. However, an increase in the $CD44^{+}CD24^{+}$ population was apparent in NMS-873 treated SKBR3 cells along with a decrease in the $CD44^{-}CD24^{+}$ population (Online Resource 3). Interestingly, when we analyzed the expression of p97/VCP mRNA levels from TCGA Breast Invasive Carcinoma samples using the cbioportal website (http://cbioportal.org) [35, 36], the levels of p97 transcript was highest in the Her2-enriched breast cancer subtype. Taken together, these findings suggest that inhibiting the processing of p100 may be effective to suppress CSC activity in Her2-enriched breast tumours.

Discussion

Through silencing of the *Nfkb2* gene in mammary cancer cell lines, we have demonstrated that concomitant loss of the *Nfkb2* gene products, p100 and p52, leads to context dependent effects on the tumorigenicity of these cells. In 4T1 cells, we observed increased NF-κB activity, induction of EMT, increased motility, CSC properties and tumorigenicity in vivo. Our results are in line with previous studies which have shown the regulation of malignant tumor characteristics and CSC properties by the NF-κB pathway [21, 33, 37, 38]. We propose that the increase in NF-κB activity after *Nfkb2* silencing may be due to the loss of p100 which plays more prominent negative regulatory roles in this cell type, suppressing both the canonical and alternative NF-κB pathways [28]. It is more likely that the consequences of p100 depletion in these cells are mediated through the canonical pathway because reconstitution with p52 did not rescue or aggravate the increased motility and CSC traits in our studies.

On the other hand, we showed that the CSC properties and tumorigenicity of N202.1A cells were positively regulated by p52. Significant decreases in CSC properties were observed in these cells upon *Nfkb2* knockdown and the sphere forming potential could be rescued by reconstitution of p52. These results coincide with a recently published study which implicated the role of the alternative NF-κB pathway in the regulation of breast CSCs [29]. Furthermore, upstream activators of the alternative NF-κB pathway such as NIK and IKKα have also been shown to positively regulate breast CSCs [39, 40]. As our results suggest a regulatory role for p100 in limiting CSC activity, the consequences of increased NIK/IKKα activity may be two-fold, losing the inhibitory function of p100 along with increased p52 activity. It is also worth noting that in the study where NIK was implicated in regulation of breast CSCs [39], examination of NIK protein levels through immunohistochemistry revealed that NIK expression

was significantly higher in the $Her2^+$ subtype and there was a positive correlation between NIK and Her2 levels. Similarly, in the study where the role of IKKα was examined in several different mouse models of breast cancer, loss of IKKα only affected tumor initiating cells in the MMTV-Neu model [40].

It is possible that not all breast cancers will exhibit a dependence on the alternative NF-κB pathway for 'stemness' and malignancy. Hence, it will be important to identify specific subtypes of breast cancer which will benefit from inhibition of the alternative NF-κB pathway. At present, it is known that NF-κB activity in general is elevated in Her2-eniched and basal subtypes of breast cancer [41, 42] but specifically, the alternative NF-κB pathway has not been associated with a more defined subset of breast cancers. Our data along with studies on upstream regulators of the alternative NF-κB pathway [39, 40] would indicate that the alternative NF-κB pathway may have a more important role in Her2-enriched breast tumors. We have also previously shown that Bcl3, a protein which can form complexes with p52 or p50 dimers to activate NF-κB, specifically plays a role in metastasis of ErbB2 driven tumors [43]. It would be interesting to address whether activation of the alternative NF-κB pathway is required for the tumor promoting properties of Bcl3.

Although the stratification of breast tumors into molecular subtypes have improved therapeutic outcomes and assisted in administration of specific therapies, current designation of tumor subtypes are based on the bulk of the tumor. Based on the CSC concept, it is possible that multiple breast cancer subtypes (Basal + Luminal) may be present within a tumour [14, 44]. With regards to that, the expression of Her2 has been shown to enrich for a luminal cancer stem cell population [45, 46] and plasticity for this $Her2^+$ CSC population has been documented, even in tumors classified as $Her2^-$ [47]. Hence it would be interesting to address whether our findings

may apply to $Her2^+$ CSC subpopulations in breast tumours which are not classified as the Her2 enriched subtype.

Importantly, the context dependent effects we observed in different mammary cancer cell lines would advocate against targeting the *Nfkb2* gene at the genetic level. Accordingly, it would be more appropriate to inhibit the processing of p100 into p52. By employing this therapeutic strategy, p52 transcriptional activity can be repressed and concomitantly, p100 levels can be maintained at higher levels. Since it is possible that p100 can also sequester canonical NF-κB subunits, targeting the processing of p100 could provide additional benefits. We have shown that inhibition of p97 can be effective in limiting CSC activity in a specific subset of breast cancer cells but other potential drug candidates include upstream kinases such as IKKα and NIK. In addition, RANKL inhibitors such as denosumab are already being employed clinically [48] and may prove to be effective in ameliorating certain breast CSCs.

It appears that after NMS-873 treatment, there was increased CD44 expression in $CD24^+$ SKBR3 cells (Online Resource 3) which was inversely associated with its sphere forming potential (Figure 6B). Although CD44 positivity is associated with BCSC phenotypes [9], it is possible that only the combined expression pattern of $CD44^+CD24^-$ enriches for BCSCs. Accordingly, previous studies of clinical samples have showed an association between CD44 positivity[49-51] and good prognosis but this observations may be convoluted because of the existence of various CD44 isoforms.

In summary, we have shown that the *Nfkb2* gene products p100 and p52, through their roles in the NF-κB pathway, regulate breast CSC properties. We found that p100 may have more significant negative regulatory effects in $4T1^{ERBB2LOW}$ cells whereas p52 activation was more

dominant in N202.1A^{ERBB2HIGH} cells. This led to contrasting effects on CSC properties and tumourigenicity upon *Nfkb2* silencing. The opposing roles of p100 and p52 in the regulation of breast CSCs gives credence to the inhibition of p100 processing into p52 as a potential strategy to target breast CSCs.

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Compliance with ethical standards

Conflict of Interest

Authors declare no conflict of interest.

Ethical Standards

The authors declare that the experiments performed in the current publication comply with the current laws of the United Kingdom and all maintenance, breeding and scientific procedures involving animals were carried out according to the guidelines set by the U.K. Home Office Regulations Animals (Scientific Procedures) Act 1986.

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Figure legends

Figure 1: Silencing of *Nfkb2* alters the basal NF-κB activity of mammary cell lines. **a** Boxplot of NFKB2 gene expression in tissue samples from TCGA breast dataset acquired through Oncomine software and based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. Horizontal bars indicate median values, box edges represent 75th and 25th percentile, error bars show 90th percentile and 10th percentile, points show outlier values. **b** Immunoblots of nuclear and cytoplasmic extracts from 4T1 and N202.1A cells showing levels of NF-κB subunits. **c** Quantification of the ratio of nuclear p52 to nuclear p100 from three independent immunoblots of 4T1 and N202.1A cells. **d** Quantification of the ratio of nuclear p52 to cytoplasmic p52 from three independent immunoblots of 4T1 and N202.1A cells. **e** Bar chart showing relative light units (RLU) from NF-κB reporter assays. Cells were transfected with NF-κB luciferase reporter and pcDNA3.1:lacZ as transfection control. 48 hours after transfection, cell lysates were harvested and the ratio of luciferase to lacZ activity measured. Bar charts show basal NF-κB activity plotted as relative light units (RLU) in the respective cell lines. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM). * indicates $p<0.05$, ** indicates $p<0.01$ and *** indicates $p<0.001$.

Figure 2: Silencing of Nfkb2 increases the motility of 4T1 cells and this increase is associated with EMT in these cells. **a** 4T1 and N202.1a cell lines transduced with non target (NT) and shRNA against Nfkb2 were plated in Boyden trans-well migration chambers and migration was induced via a serum gradient. Bar charts show the relative number of cells migrated (normalized

to 4T1 NT). **b** 4T1 NT and 4T1 Nfkb2 kd cells were plated in Boyden trans-well migration chambers in the presence or absence of 1uM BAY 11-7082 and migration was induced via a serum gradient. Bar charts show the number of cells migrated. **c** 4T1 cells transduced with non target (NT) shRNA, shRNA against Nfkb2 and shRNA against Nfkb2 with p52 over-expression were plated in Boyden trans-well migration chambers and migration was induced by a serum gradient. Bar charts show number of cells migrated normalized against NT control. **d** qPCR analysis of transcripts from 4T1 NT and 4T1 Nfkb2 kd cells. Levels of EMT inducing transcription factors were quantified as relative levels of transcripts normalized against NT controls using the ΔΔCT method. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM). * indicates $p<0.05$, where statistical significance relative to NT controls were determined by two-tailed t-test.

Figure 3: Effects of silencing *Nfkb2* on the proportion of putative CSCs in mammary cancer cell lines. **a** Bar chart showing the percentage of CD29+ CD61+ CSCs in 4T1 and N202.1A cell lines transduced with non target (NT) or shRNA against *Nfkb2*. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM), *** indicates $p<0.001$, where statistical significance relative to NT controls were determined by two-tailed t-test. Representative dot plots showing percentage of CD29+ CD61+ CSCs in **(b)** 4T1 and **(c)** N202.1A cell lines transduced with non target (NT) or shRNA against *Nfkb2*.

Figure 4: Effects of silencing *Nfkb2* on the CSC properties of mammary cell lines. **a** 4T1 and N202.1A cell lines transduced with non target (NT) or shRNA against *Nfkb2* were plated in non-

adherent culture plates with mammosphere media. Bar charts show % mammosphere forming units (MFUs) in Passage 2 calculated as the number of spheres per cells seeded. **(b)** 4T1 and **(c)** N202.1a cell lines in the first passage (P1) with or without 1uM BAY 11-7082 were dissociated and re-seeded at an equal density (P2) under non-adherent culture conditions in mammosphere media. The number of spheres formed in the presence or absence of BAY 11-7082 respectively after 7 days were counted. **(d)** 4T1 and **(e)** N202.1a cells transduced with non target (NT) shRNA, shRNA against *Nfkb2* and shRNA against *Nfkb2* with p52 over-expression were plated in non-adherent culture plates with mammosphere media and the number of spheres formed after 7 days in passage 2 was counted. Bar charts show the number of spheres formed. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM). * indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$, n.s. indicates not significant, where statistical significance relative to NT controls were determined by two-tailed t-test.

Figure 5: Effects of silencing Nfkb2 on the tumorigenic potential of mammary cancer cells in vivo. **a** 10,000 4T1 NT or Nfkb2 kd cells were transplanted into the right mammary fat pads of Balb/C mice respectively. Line graphs show the tumour growth kinetics of respective cell lines. * indicates $p<0.05$, where statistical significance between growth curves was determined by ANCOVA test; $F=8.370$, $p=0.018$. **b** Representative images from 4T1 NT and Nfkb2 kd tumours immuno-labelled with antibodies against Ki67 and cleaved caspase-3 respectively. Scale bar represents 100um. **c** Bar charts showing number of Ki67 positive nuclei per 10x field of view. **d** Bar charts showing number of cleaved caspase-3 positive cells per 10x field of view. **e** 50,000 N202.1A NT or Nfkb2 kd cells were transplanted into the right mammary fat pads of NOD/SCID mice respectively. The resulting tumours were measured and the growth kinetics plotted as line

graphs. Data points represent averages from n=5 animals per cohort and error bars represent standard error of the mean. * indicates $p<0.05$, where statistical significance between growth curves was determined by ANCOVA test; $F=7.754$, $p=0.021$. **f** Representative images from N202.1a NT and Nfkb2 kd tumours immuno-labelled with antibodies against Ki67 and cleaved caspase-3 respectively. Scale bar represents 100um. **g** Bar charts showing number of Ki67 positive nuclei per 10x field of view. **h** Bar charts showing number of cleaved caspase-3 positive cells per 10x field of view.

Figure 6: NMS-873 inhibits the sphere forming potential of ErbB2 high mammary cancer cells. **a** 4T1 and N202.1A cell lines transduced with non target (NT) or shRNA against *Nfkb2* were plated in non-adherent culture plates with mammosphere media in the absence or presence of 1μM NMS-873. Bar charts show sphere forming efficiency in Passage 2 calculated as the number of spheres per cells seeded. **b** Bar charts showing the sphere forming efficiency of MDA-MB-231 and SKBR3 cells in the absence or presence of 1μM NMS-873. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM). ** indicates $p<0.01$, *** indicates $p<0.001$, where statistical significance relative to NT controls were determined by two-tailed t-test. **c** Box plots showing the levels of VCP/p97 mRNA levels in patients from the Breast Invasive Carcinoma TCGA cohort grouped according to PAM50 subtypes. Data was obtained using the cbioportal website and based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/. Statistical significance was determine using ANOVA test; $F=8.69$, $p<0.0001$.

Online Resorce 1: Silencing of Nfkb2 does not affect the proliferation and colony forming potential of mammary cancer cell lines. **a** Immnoblot showing the levels of p100 and p52 in 4T1 and N202.1A cells after transduction with Non-Target (NT) or Nfkb2 shRNA. Growth curves showing the number of cells over time for **(b)** 4T1 and **(c)** N202.1A cells. **d** Colony forming assay of 4T1 and N202.1A cells seeded in 6-well plates at a density of 1000 cells/well and quantified after 7 days. Data points represent average of at least n=6 and error bars indicate standard error of the mean (SEM). N.S. indicates a non significant difference, where statistical significance relative to NT controls were determined by two-tailed t-test.

Online Resorce 2: Silencing of Nfkb2 with independent shRNAs in 4T1 cells. **a** Immunoblot showing levels of p100 and p52 after knockdown with non target, p52 sh43 or p52 sh44 in 4T1 cells. **b** Bar chart showing number of migrated cells in a 10x field of view for Boyden chamber migration assays of 4T1 non target, p52 sh43 or p52 sh44 cells. **c** Bar chart showing number of spheres formed for mammosphere forming assays of 4T1 non target, p52 sh43 or p52 sh44 cells.

Online Resorce 3: Effects of NMS-873 treatment on CSC marker expression in human breast cancer cell lines. **a** Representative dot plots showing expression of CD44+ and CD24+ in MDA-MB-231 and SKBR3 cell lines treated with vehicle or 1μM NMS-873 for 48 hours under normal culture conditions and analyzed by flow cytometry. Bar charts showing the percentage of **b** $CD44^{+}CD24^{-}$, **c** $CD44^{+}CD24^{+}$, or **d** $CD44^{-}CD24^{+}$ populations. Data points represent average of

at least n=6 and error bars indicate standard error of the mean (SEM), ** indicates $p<0.01$, where statistical significance relative to vehicle controls were determined by two-tailed t-test.

Figure 1

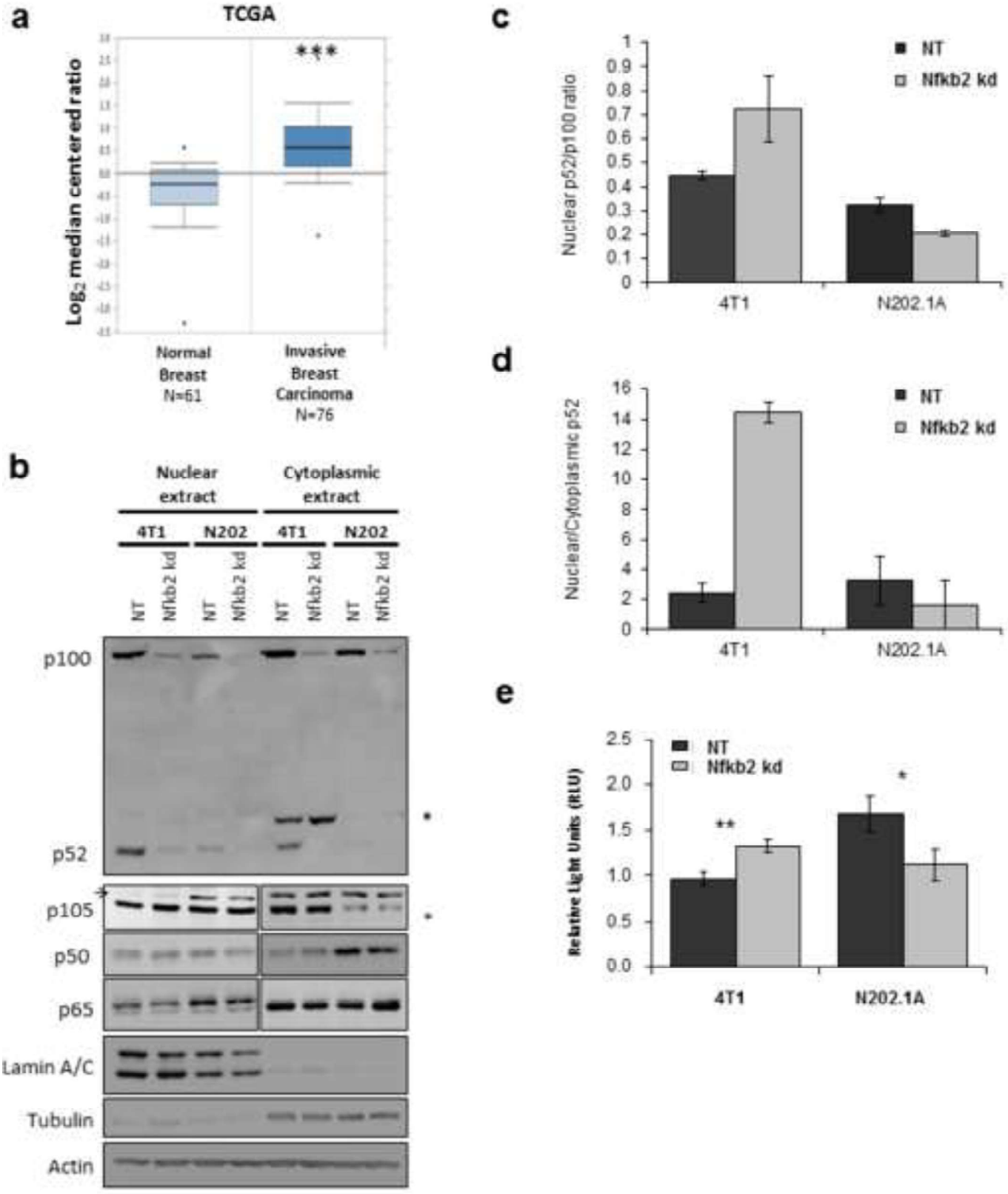
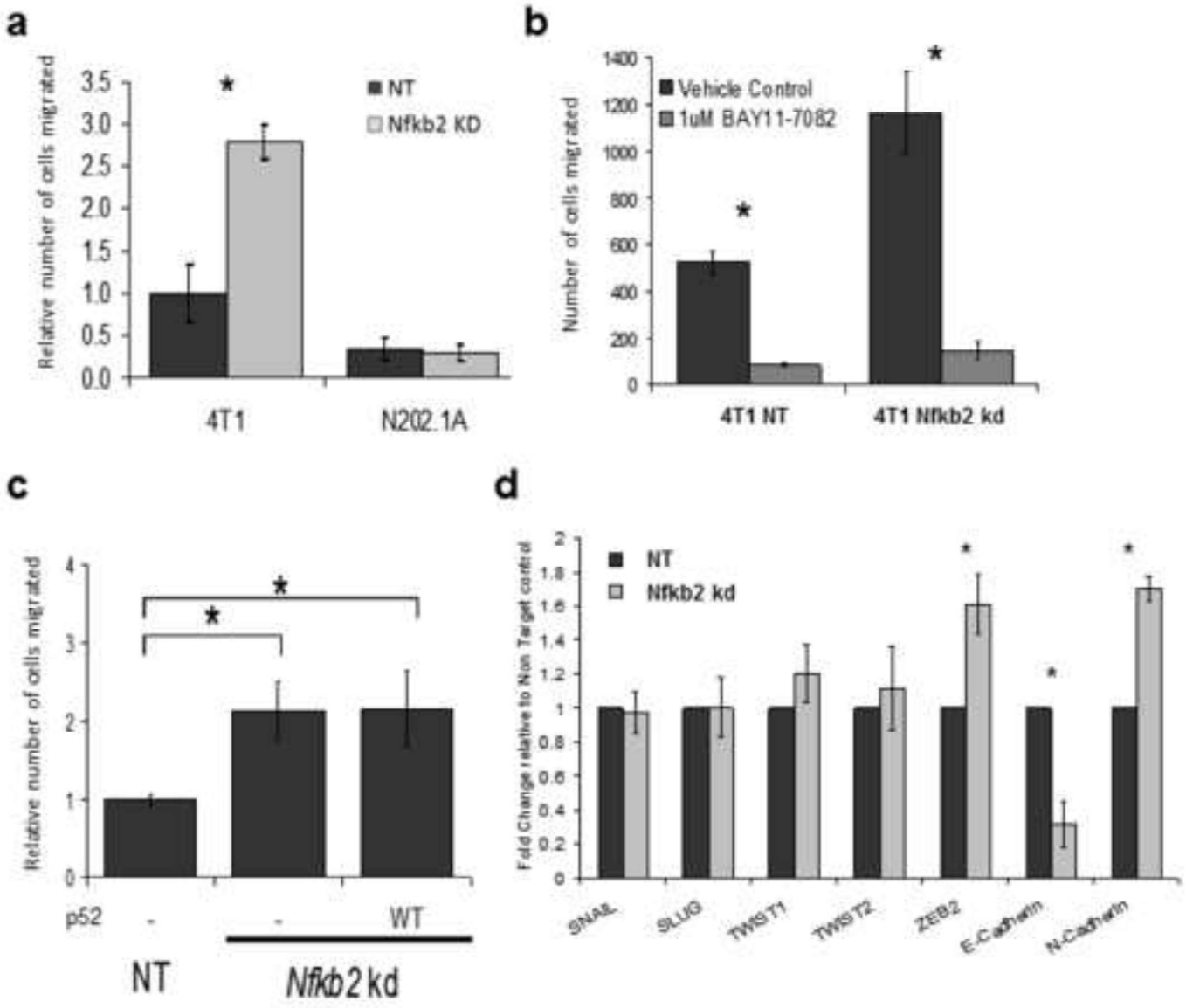


Figure 2



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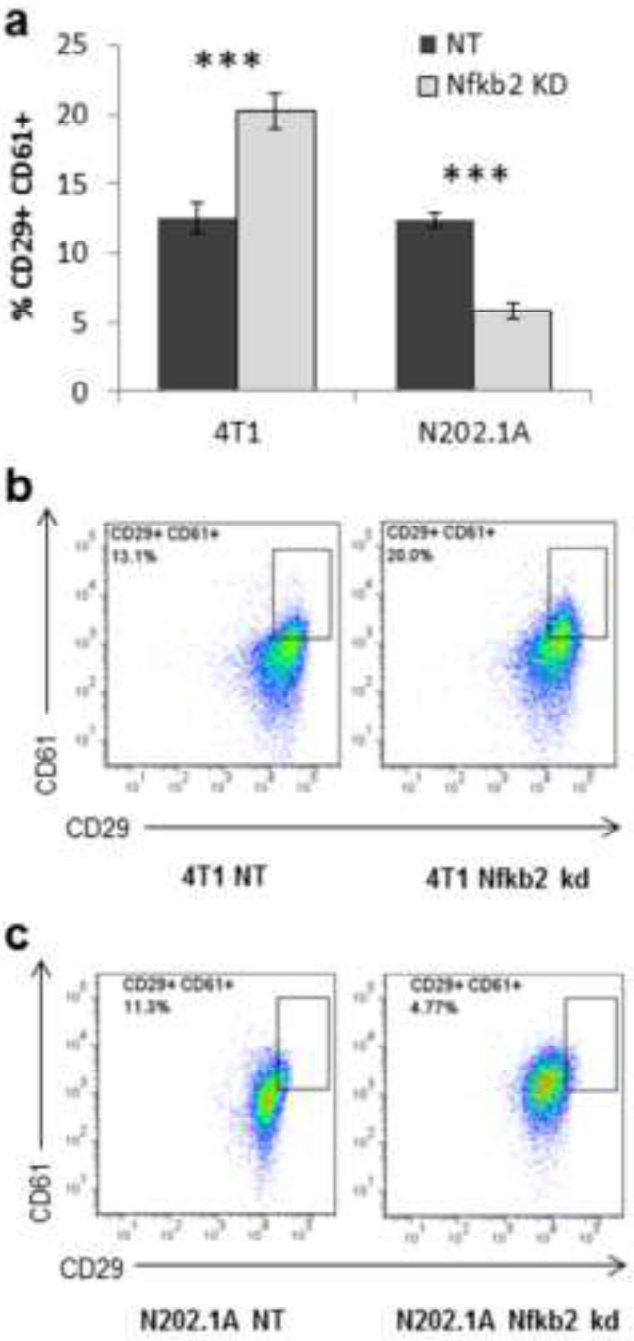

Figure 4

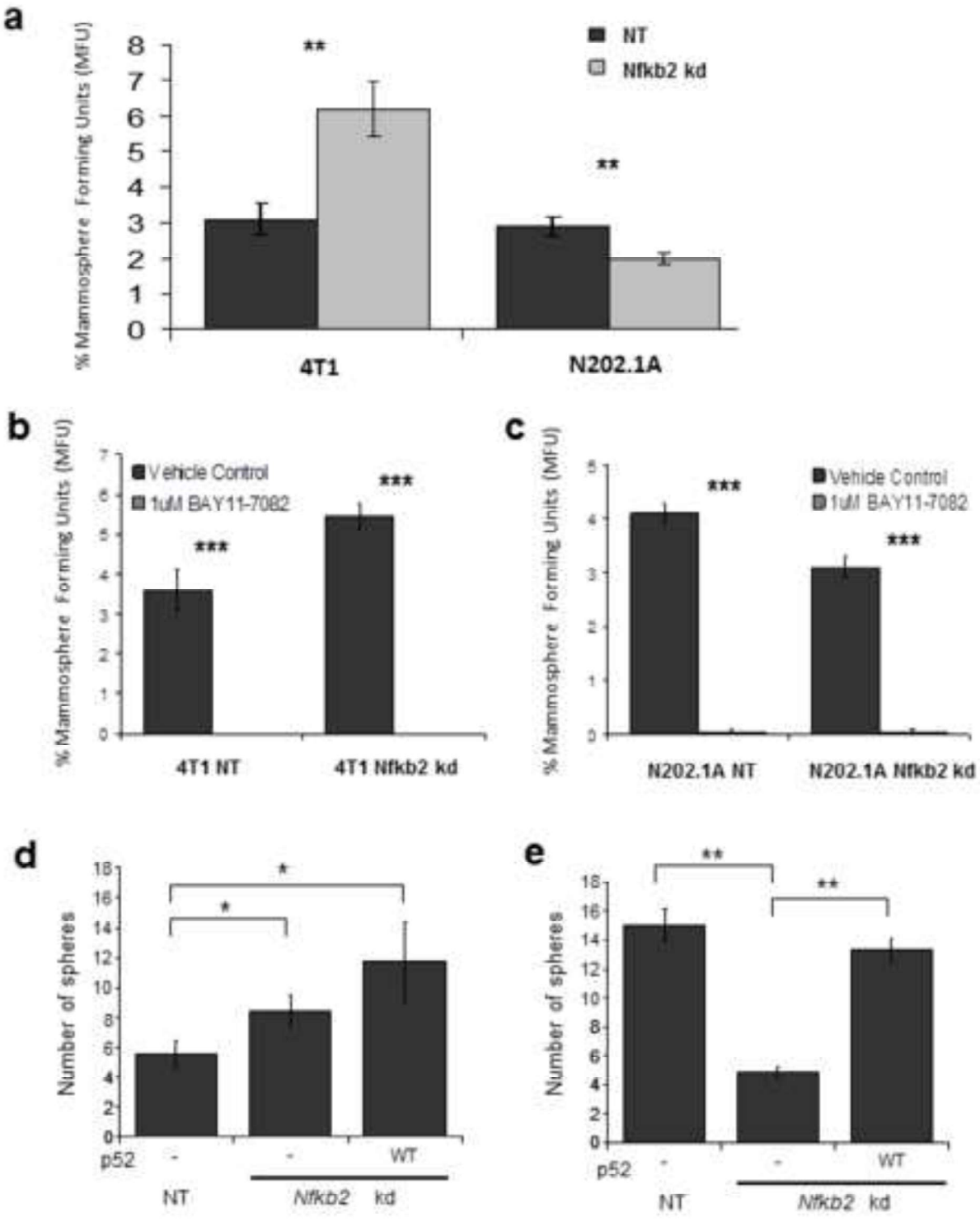

Figure 4

Figure 5

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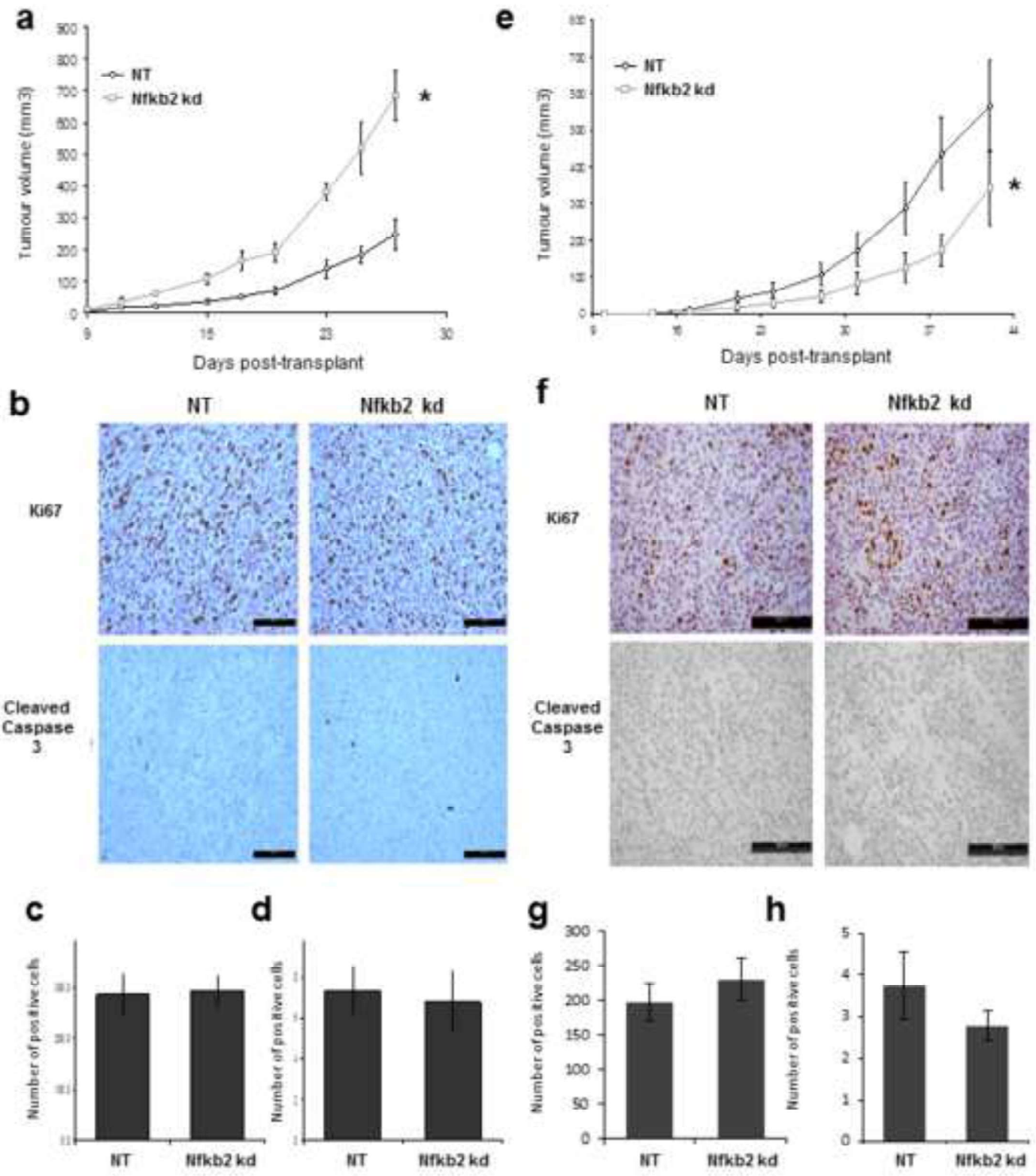

Figure 5

Figure 6

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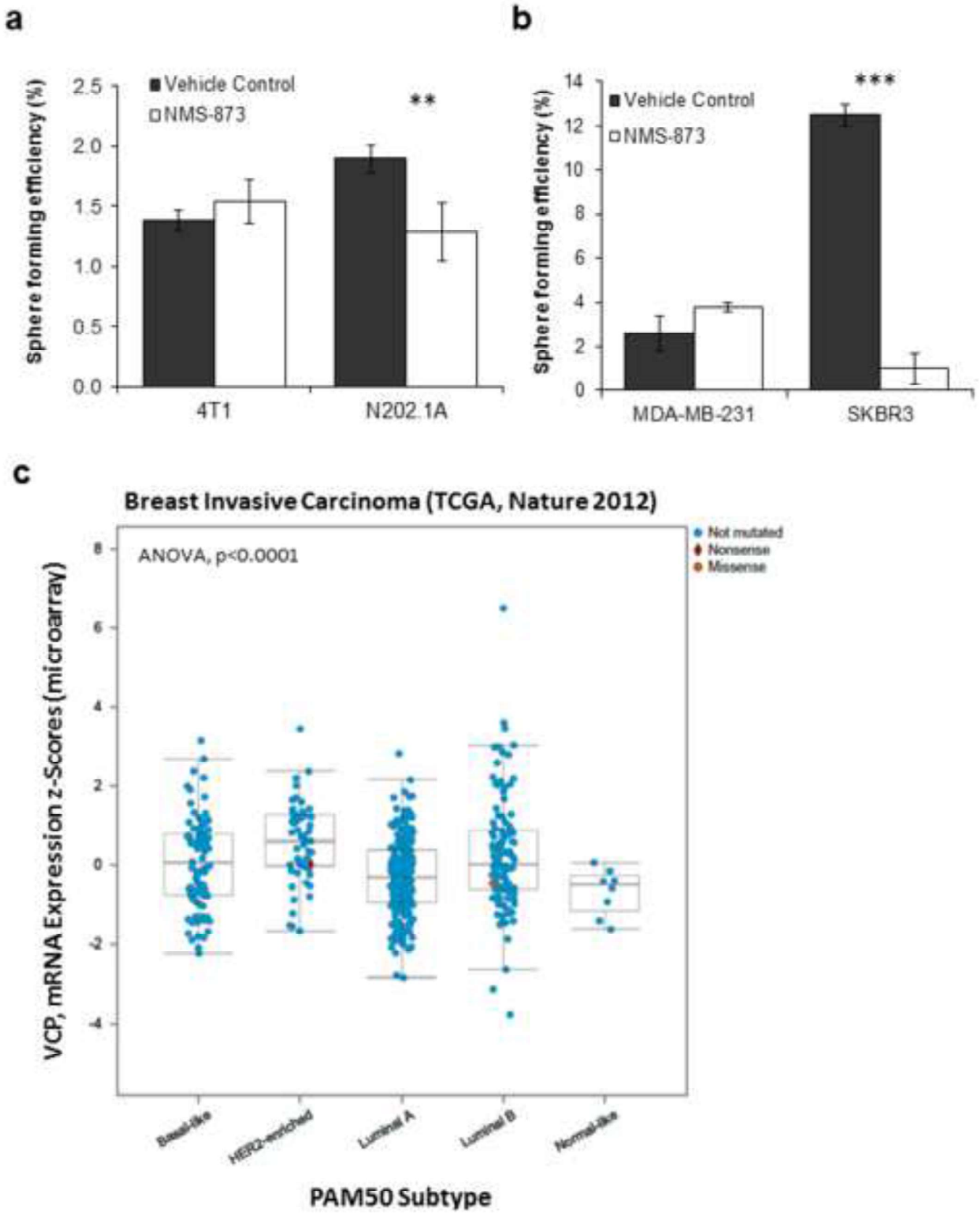

Figure 6